ORIGINAL ARTICLE 10.1111/j.1469-0691.2004.00898.x

Pneumonia caused by *Mycobacterium kansasii* in a series of patients without recognised immune defect

S. M. Arend[1], *E. Cerdá de Palou*[2], *P. de Haas*[3], *R. Janssen*[1], *M. A. Hoeve*[4], *E. M. Verhard*[1], *T. H. M. Ottenhoff*[4], *D. van Soolingen*[3] *and J. T. van Dissel*[1]

[1]Department of Infectious Diseases, Leiden University Medical Center, Leiden, [2]Regional Health Service (Gemeenschappelijke Gezondheids Dienst), Southeast Limburg, [3]Department of Mycobacteria, National Institute of Public Health and the Environment (Rijks Instituut voor Volksgezondheid en Milieu), Bilthoven and [4]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

ABSTRACT

The clinical and epidemiological characteristics of 17 patients diagnosed with *Mycobacterium kansasii* pneumonia within a limited geographical region over a period of 10 years are described. An in-depth evaluation of the innate and adaptive immune systems was performed for five available patients. A comparison was made of the genetic fingerprint patterns of the isolates obtained by restriction fragment length polymorphism (RFLP) analysis, with the major polymorphic tandem repeat (MPTR) as a probe. Predisposing factors consisted of smoking, airway abnormalities, substance abuse, diabetes or poor general condition, but in two patients no risk factor was identified. In the five patients tested, no abnormalities or deficiencies were detected in the innate or adaptive type-1 immunity. All *M. kansasii* isolates had identical MPTR RFLP patterns, although no epidemiological connection could be established, and these were identical to those of clinical isolates from Australian patients. These data do not support the theory that defects in the innate or adaptive type-1 immunity have a role in the pathogenesis of invasive *M. kansasii* infections. The identical fingerprint patterns of the isolates suggested the existence of a virulent strain of *M. kansasii*.

Keywords Cellular immune system, genotyping, *Mycobacterium kansasii*, pneumonia, risk factors, tuberculosis

Original Submission: 11 August 2003; **Revised Submission:** 18 September 2003; **Accepted:** 13 November 2003

Clin Microbiol Infect 2004; 10: 738–748

INTRODUCTION

Mycobacterium kansasii can cause lung infections in apparently healthy subjects, unlike most other environmental mycobacterial species which cause illness almost exclusively in subjects with defects in the cellular immune system as a result of disease or immunosuppressive treatment [1]. *M. kansasii* pneumonia mimics classical lung tuberculosis in many respects, which could reflect similarities in the pathogenesis of these infections caused by shared virulence factors of these mycobacteria and similarities in host immunity and susceptibility [2,3]. The relative importance of characteristics of the microorganism, the host and the environment in the pathogenesis of invasive infection with *M. kansasii* in immunocompetent subjects has not so far been elucidated.

The significant geographical variations seen in the incidence of *M. kansasii* pneumonia, which cannot be deduced simply from latitude or climate, suggest that the environment is a relevant factor [4,5]. A relatively high incidence of *M. kansasii* pneumonia has been noted in certain parts of the USA and in highly industrialised regions of central Europe, the south-east of the UK and the southern part of The Netherlands, all of which were, at that time, mining and industrial areas with heavy air pollution [6–9]. The natural reservoir of *M. kansasii* is largely

Corresponding author and reprint requests: S. M. Arend, Department of Infectious Diseases, C5P, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands
E-mail: s.m.arend@lumc.nl

unknown, but has been postulated to include water systems associated with habitation or industry, since *M. kansasii* can be isolated from such systems [10].

Extrapulmonary or disseminated *M. kansasii* infections have been observed mainly in subjects with compromised cellular immunity [4], including a small number of patients with mendelian disorders of the interferon gamma (IFN-γ) receptor [9,11–13]. Invasive lung infection with *M. kansasii* in the absence of a cellular immune defect is often related to structurally abnormal airways, associated with chronic obstructive pulmonary disease, bronchiectasis or decreased mucociliary clearance as a result of smoking or exposure to heavy air pollution [9]. Particularly when *M. kansasii* pneumonia occurs in an individual without airway abnormalities and known immune defects, the suspicion of an underlying but as yet unrecognised immune defect arises. However, detailed studies on type-1 cell-mediated immunity against mycobacteria have not been reported previously in patients suffering from isolated pulmonary infection with *M. kansasii*.

An unusually high number of patients with *M. kansasii* pneumonia has been observed previously in a former mining area in The Netherlands [14]. The occurrence of this infection in a young man with no risk factors for this infection prompted an evaluation of all patients with documented *M. kansasii* pneumonia who had been reported at one regional health centre over a 10-year period. This study describes the risk factors, clinical presentation and epidemiological investigation of 17 patients with *M. kansasii* pneumonia. All causative *M. kansasii* isolates were genotyped and fingerprint patterns were compared. The innate and adaptive cellular immune systems of five available patients were studied in detail.

PATIENTS AND METHODS

Patients

Patients were eligible if they had been diagnosed between 1991 and the end of 2001 with *M. kansasii* pneumonia in the absence of a recognised immune defect, such as infection with HIV, treatment with corticosteroids, other immunosuppressive agents or chemotherapy. Although there is no central registry in The Netherlands for *M. kansasii* infections, positive mycobacterial cultures are generally sent to the National Institute of Public Health and the Environment in Bilthoven for species identification and drug susceptibility testing. After adjustment for multiple cultures/patient, an unusually high number of isolates appeared to originate from a limited geographical region in the southern part of The Netherlands, namely the former mining area in the southeastern part of the province of Limburg. Patients from that region were recruited if they had been recorded as suffering from *M. kansasii* pneumonia by the Regional Health Authority in South Limburg. To discriminate infection from colonisation, *M. kansasii* infection was defined according to the criteria used by the American Thoracic Society (ATS) [15]. In brief, these criteria require characteristic radiographic abnormalities in combination with at least two positive sputum cultures, plus one smear positive for acid-fast bacilli, or three positive sputum cultures within 12 months, or at least growth graded as 2+ from a bronchial wash, or characteristic histology in a lung biopsy and at least one positive culture, in the absence of an alternative diagnosis.

Demographic and clinical characteristics obtained retrospectively from the charts included age, sex, date of diagnosis, profession, smoking habits, previous lung disease, extent and results of contact investigations, presence or absence of systemic symptoms, radiographic findings, result of acid-fast staining, type of specimens cultured and number of positive cultures, histology, duration and type of treatment and outcome. Tuberculin skin test results and the results of skin testing using tuberculin from *M. kansasii* (obtained from the National Institute of Public Health and the Environment; the availability of this product ceased in January 2003) were recorded if these tests had been performed.

Genotyping of *M. kansasii* isolates and fingerprinting by major polymorphic tandem repeats

M. kansasii isolates were typed by restriction fragment length polymorphism (RFLP) analysis, with the major polymorphic tandem repeat (MPTR) as a probe and *Pvu*II as the restriction enzyme [16]. Computer-assisted analysis of the MPTR patterns was carried out with BioNumerics v. 3.0 (Applied Maths, St Martens Latem, Belgium). The INNO-LiPA Mycobacteria DNA probe test (Innogenetics, Ghent, Belgium), targeting the 16S–23S rRNA internally transcribed spacer sequence, was used to identify *M. kansasii* internally transcribed spacer sequence genotypes as described previously [17]. For comparison, one *M. tuberculosis* reference strain, two other clinical *M. kansasii* isolates from patients in other regions within The Netherlands, 20 isolates from Australian patients (kind gift of B. Dwyer, Clinical Pathology Laboratory, Fairfield Infectious Diseases Hospital, Victoria, Australia), isolates from seven Dutch patients who did not fulfil the ATS criteria and in whom the isolation of *M. kansasii* was considered to reflect colonisation, and three isolates of environmental origin, were also tested with the INNO-LiPA mycobacteria test.

Immunological investigation

Blood was obtained for immunological evaluation from five patients who remained alive and could be contacted at the time of this study. The protocol (P207/99) was approved by the Institutional Review Board of Leiden University Medical Center. Five heparinised tubes (9 mL each) and two lipopolysaccharide-free tubes (4 mL each) of venous blood were obtained by standard methods. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation and tested immediately or frozen in RPMI medium (Gibco, Paisley,

UK) containing fetal calf serum 20% v/v and dimethylsulphoxide 20% v/v for later use. Whole blood assays were started on the day of blood sampling. For comparison, blood cells obtained from healthy non-BCG-vaccinated blood donors were included in all assays. Written informed consent was obtained from all patients and control subjects.

Antigen-stimulated interferon-γ production

Antigen-specific T-cell responses to various mycobacterial antigens were evaluated by 6-day PBMC cultures, with IFN-γ production being measured with an ELISA technique (Ucy-Tech, Utrecht, The Netherlands), as well as by 20-h PBMC cultures for enumeration of IFN-γ-producing antigen-specific T-cells with an enzyme-linked immunospot assay (ELISPOT). The following antigens were tested: purified protein derivative (PPD), *M. tuberculosis* sonicate, short-term culture filtrate, *M. avium* sonicate, and the common mycobacterial antigen Ag85B. The source of these antigens, the concentrations used, the culture techniques and measures of T-cell activation have been described elsewhere [18].

FACS analysis

Fresh PBMCs were washed with phosphate-buffered saline containing bovine serum albumin 0.2% w/v and incubated with phycoerythrin-conjugated monoclonal antibodies directed against IFN-γ receptor 1 (IFN-γR1) (Genzyme, Cambridge, MA, USA), IFN-γR2, tumour necrosis factor alpha receptor 1 (TNF-αR1) or TNF-αR2 (Caltag Laboratories, Burlingame, CA, USA), and fluorescein isothiocyanate-conjugated antibodies directed against CD14 (DAKO, Glostrup, Denmark). For each sample, 10^4 cells were analysed with a FACScalibur (Becton Dickinson, Mountain View, CA, USA). In addition, receptor expression on CD14-positive cells was determined.

To generate phytohaemagglutinin T-cell blasts, PBMCs (10^6 cells/mL) were incubated for 10 days in 24-well plates in standard Iscoves Modified Dulbecco Medium (IMDM; Life Technologies, Paisley, UK) supplemented with pooled complement-inactivated human serum 10% v/v, phytohaemagglutinin 2 μg/mL and recombinant interleukin-2 (IL-2) (Chiron, Amsterdam, The Netherlands) at a final concentration of 25 U/mL. Cell surface expression of IL-12Rβ1 and IL-12Rβ2, IL-18R and IFN-γR1 by phytohaemagglutinin blasts was analysed by labelling $0.5–2 \times 10^5$ T-cell blasts 3 days after stimulation, followed by measurement on a FACScalibur, as described previously [19,20].

Stimulation of whole blood

Blood collected in endotoxin-free tubes (Endotube ET; Chromogenix, Milan, Italy) was diluted five to ten-fold in IMDM and stimulated for 18 h with 1–100 ng/mL lipopolysaccharide, *M. tuberculosis* or *M. avium* sonicate (final concentration 10 μg/mL; a kind gift of A. Kolk, Royal Tropical Institute, Amsterdam, The Netherlands) in the presence or absence of IFN-γ (5–5000 IU/mL). Levels of IL-12 p40, IL-12 p70 (R & D Systems, Abingdon, UK), IL-10, IL-6 and TNF-α (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) were determined by ELISA in pooled supernatants of triplicate wells. TNF-α response was analysed by incubating whole blood diluted five-fold with increasing amounts of TNF-α (10–100 ng/mL) and measurement of IL-6 production in supernatants after culture for 18 h.

IL-12 and IL-18 responsiveness of T-cells

Responsiveness to IL-12, IL-18 and combinations of these was determined by stimulation of 5×10^4 phytohaemagglutinin blasts for 72 h with mitogenic antibodies in the presence or absence of recombinant human IL-12 (final concentrations 40, 200 and 1000 pg/mL; R & D Systems) or IL-18 (final concentration 50 ng/mL; Medical and Biological Laboratories, Nagoya, Japan) or in the presence of both, as described previously [21]. IFN-γ levels in pooled supernatants of triplicate wells were determined by ELISA. The cultures were incubated in the presence of (^{3}H)thymidine (0.5 μCi/well) for an additional 16 h to measure cellular proliferation.

IFN-γ responsiveness of monocytes

PBMCs were incubated overnight in hydron-coated wells to prevent adherence of the monocytes, with different concentrations of IFN-γ (0–100 IU/mL). Cells were washed and stained with fluorescein isothiocyanate-conjugated anti-CD64 and phycoerythin-conjugated anti-CD14. CD64 expression on CD14-positive cells was analysed with a FACScalibur, as described above.

Statistical analysis

The results of the immunological assays in patients and control subjects were compared non-parametrically with the Mann–Whitney test.

RESULTS

Clinical characteristics of patients with *M. kansasii* pneumonia

Between May 1991 and August 2001, 17 patients diagnosed with *M. kansasii* pneumonia according to the ATS criteria had visited the Regional Health Authority in South Limburg at least once during the course of their treatment. Tables 1 and 2 describe the demographic and clinical characteristics of these patients. The mean age at diagnosis was 50.6 ± 14.3 years, and 13 (76%) patients were men. All patients were native Dutch, except patient 8, who was born in Indonesia. Although the profession was not retrievable for all patients, the work of five patients (4, 5, 7, 9 and 15) involved intensive contact with water systems. No predisposing factor could be identified in patients 12 and 16.

Two patients were asymptomatic at the time of diagnosis, which was made following routine chest radiography. The remaining 15 patients had been symptomatic for a mean of 3.9 ± 3.1 months

Table 1. Characteristics of 17 patients with *Mycobacterium kansasii* pneumonia

No.	Sex	Age at diagnosis (years)	Year of diagnosis	Profession	Smoking (cig./day)	Other predisposing factors	Special characteristics
1	F	61.2	1991	House-keeper	> 20	COPD	
2	M	70.3	1991	Retired (unknown)	NA	Poor general condition	
3	M	63.9	1991	Graphic designer	20	COPD	
4	M	62.7	1992	Farmer	30	DM, alcohol	
5[a]	M	39.6	1992	Plumber	5–10	Recurrent airways infections	
6	M	48.9	1992	Bus driver	> 25	COPD	
7	M	50.2	1993	Sand-blaster	25	None	
8	M	37.4	1993	Retired (unknown)	> 25	COPD, drugs	
9[a]	M	44.7	1993	Central heating worker	15	COPD	
10	M	35.2	1993	Jobless	> 25	Drugs, alcohol, bronchitis	
11	F	35.8	1993	Administrator	> 20	Recurrent pneumothorax	
12[a]	M	47.7	1995	Administrator	0	None	Marathon runner
13[a]	M	71.5	1997	Retired administrator	15	None	
14	M	76.3	1999	Retired mine worker	0	COPD, silicosis	
15	M	44.3	2000	Laundry worker	25	COPD	
16[a]	F	39.5	2000	Primary school teacher	0	None	Nail–patella syndrome
17	F	31.4	2001	Bank employee	25	None	

COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus.
[a]Patient who participated in the immunological evaluation.

Table 2. Clinical features of 17 patients with *Mycobacterium kansasii* pneumonia

No.	Symptoms (months)	Haemoptysis	Weight loss	Fever	Chest X-ray[b]	Cavities	TST (mm)	PPD-K skin test (mm)	Ziehl–Neelsen	Culture of specimen (no. of positive cultures); histology with granulomas (+)
1	2		+		1		15	ND	+	Sputum (3)
2	3		+	+	2		ND	ND	+	Sputum (2)
3	6		+		2		ND	ND	+	Sputum (2)
4	None				1	+	ND	ND	+	BAL and sputum (4); histology (+)
5[a]	5	+	+	+	2	+	10	20	+	Sputum (5)
6	12	+	+	+	2	+	10	15	+	BAL and sputum (3)
7	0.5		+	+	2	+	0	0	+	Sputum (2)
8	6		+	+	2	+	ND	ND	+	Sputum (> 1, not quantified)
9[a]	1			+	2	+	ND	ND	–	Biopsy and sputum (3); histology (+)
10	2	+		+	1	+	0	5	+	BAL and sputum (3)
11	None				1		0	0	+	BAL and biopsy (2); histology (+)
12[a]	1.3		+		1	+	0	8	+	BAL and sputum (10)
13[a]	6	+	+		1	+	ND	ND	+	Sputum (3)
14	4.5				2	+	ND	ND	+	Sputum (3)
15	2				2	+	40	ND	+	Sputum (3)
16[a]	6				1	+	25	ND	+	Sputum (5)
17	1		+	+	1		15	ND	+	BAL and sputum (7)

BAL, bronchoalveolar lavage; PPD-K, tuberculin of *M. kansasii*; TST, tuberculin skin test; ND, not done.
[a]Patient who participated in the immunological evaluation.
[b]One- or two-sided infiltrates.

(median 3; range 0.5–12). All symptomatic patients complained of coughing, accompanied in some by haemoptysis (4/15 patients), weight loss (10/15) and/or fever (7/15). Total lymphocyte counts were normal (> $10^3/\mu L$) at the time of diagnosis. All patients fulfilled the ATS criteria for the type and number of positive cultures, with a mean of 3.6 (range 2–10) positive cultures/patient (Table 1). Initial treatment consisted of isoniazid, rifampicin and pyrazinamide, with the latter being replaced with ethambutol, clarithromycin or a fluoroquinolone after culture results were known, for a total mean duration of treatment of 13.1 ± 4.6 months. One patient died during treatment. None of the 17 patients had been included in any of the other patients' contact investigations. Also, no temporal or spatial epidemiological link could be identified retrospectively between the patients.

M. kansasii genotyping

The internally transcribed spacer sequence genotypes were determined and MPTR RFLP typing was performed to assess the evolutionary lineage of these clinically significant *M. kansasii* isolates. All 17 clinical isolates from this study were of the INNO-LiPA MKA-I type. The clinical isolates from other regions in The Netherlands and Australia were also of the INNO-LiPA MKA-I

type, except one isolate that was INNO-LiPA MKA-II. One of the three environmental isolates was INNO-LiPA MKA-II, and two were INNO-LiPA MKA-III.

Fig. 1 shows the MPTR RFLP patterns of the 15 patient isolates available for typing (the isolates of patients 2 and 4 had insufficient growth for fingerprinting), demonstrating that the patterns were identical. For clarity, only some of the control strains are shown: two Dutch clinical isolates from another study (one MKA-I and one MKA-II), two of the 20 clinical isolates from Australia (all isolates were MKA-I and had identical MPTR patterns), three environmental *M. kansasii* strains and one *M. tuberculosis* strain. Six of seven isolates from patients colonised with *M. kansasii* were MKA-I, and one was MKA-II (not shown).

Interestingly, all INNO-LiPA MKA-I isolates had identical MPTR patterns irrespective of the geographical origin, suggesting a clonal origin of the causative *M. kansasii* isolates. The exception, which was of the INNO-LiPA type MKA-II, showed a strongly deviant MPTR RFLP pattern, which was identical to that of the INNO-LiPA MKA-II environmental isolate from another geographical region.

Evaluation of innate and adaptive cell-mediated immunity

In view of the identical fingerprint patterns of the causative *M. kansasii* strains in the absence of a common source of exposure, it was assumed that this particular strain was widespread in the environment in that region, with exposure being rather common. If that were true, these patients could have become ill either because of exposure to a large inoculum or because they had an unrecognised cellular immune defect. The lack of knowledge about the source of infection precluded the study of circumstances affecting inoculum size. Since individuals with defects in type-1 immunity are highly susceptible to infection with non-tuberculous mycobacteria [11], type-1 immune responses and the expression of relevant cytokine receptors were investigated in patients with *M. kansasii* infection. For this purpose, blood

MKA type	Source	Country of origin	Patient no.
II	Human	The Netherlands	
II	Environment	Italy	
III	Water	Belgium	
III	Biofilm	Germany	
I	Human	The Netherlands	1
I	Human	The Netherlands	3
I	Human	The Netherlands	5
I	Human	The Netherlands	6
I	Human	The Netherlands	7
I	Human	The Netherlands	8
I	Human	The Netherlands	9
I	Human	The Netherlands	10
I	Human	The Netherlands	11
I	Human	The Netherlands	12
I	Human	The Netherlands	13
I	Human	The Netherlands	14
I	Human	The Netherlands	15
I	Human	The Netherlands	16
I	Human	The Netherlands	17
I	Human	The Netherlands	
I	Human	Australia	
I	Human	Australia	
	M.tb reference	The Netherlands	

Fig. 1. RFLP fingerprint patterns of *Mycobacterium kansasii* isolates with the major polymorphic tandem repeat (MPTR) as a probe and *Pvu*II as restriction enzyme. The INNO-LiPA Mycobacteria test was used to identify the *M. kansasii* internally transcribed spacer sequence genotypes (see Methods), indicated by MKA type I, II or III. The source and origin of each isolate are indicated. Patient number refers to the numbering as used in Table 1. For comparison, the results of one *M. tuberculosis* reference strain, two other clinical *M. kansasii* isolates from patients in other regions within The Netherlands (one MKA-I and one MKA-II) and two from Australian patients (MKA-I), as well as three isolates of environmental origin, are shown. The MTPR pattern of the Australian isolates was not different from that of the other MKA-I isolates; the difference in quality of the bands was explained by prolonged storage of the DNA.

was obtained from five available patients (patients 5, 9, 12, 13 and 16 in Table 1, thus including both patients with no recognised risk factors). No selection criteria were used, other than availability and consent. The patients available for immunological evaluation did not differ significantly from untested patients with regard to age, sex or extent of disease.

Antigen-specific immune responses

IFN-γ production by PBMCs obtained from subjects infected with *M. kansasii* in response to *M. tuberculosis* sonicate, *M. avium* sonicate or Ag85B did not differ from the responses of PBMCs from persons infected with *M. marinum* or *M. tuberculosis*, or of healthy controls (Table 3). IFN-γ responses to PPD and short-term culture filtrate of *M. tuberculosis* were significantly higher in tuberculosis patients than in healthy controls and patients infected with *M. kansasii*. IFN-γ ELISPOT results in response to PPD were higher in tuberculosis patients, but were not statistically different between groups. These findings reflect the broad cross-reactivity of immune responses to complex antigens such as PPD or mycobacterial sonicates, or to a recombinant protein antigen (Ag85B) that has a very high level of homology between mycobacterial species [22]. Together, these results gave no indication of decreased antigen-specific IFN-γ responses to mycobacterial antigens.

FACS analysis of cytokine receptor expression

Monocytes of all five patients expressed normal levels of IFN-γR1, IFN-γR2, TNF-αR1 and TNF-αR2, when compared with healthy controls. Similarly, the expression of IL-12Rβ1, IL-12Rβ2 and IL-18R on activated T-cell blasts of the patients was not different from that of controls (data not shown).

Evaluation of cell-mediated immunity

The functional responses to various cytokines and stimuli of the innate immune system were evaluated by incubating whole blood with lipopolysaccharide or mycobacterial sonicates, in the presence or absence of IFN-γ. The resulting levels of TNF-α and IL-10 were in the same range for patients and controls (Table 4). IFN-γ-induced up-regulation of TNF-α expression and down-regulation of IL-10 production by lipopolysaccharide-stimulated cells were normal (data not shown). PBMCs were incubated with increasing amounts of IFN-γ, and the expression of CD64, which is regulated by IFN-γ, was measured. All five patients showed a dose-dependent up-regulation of CD64 expression within the normal range (Table 4).

The levels of IL-6 production in whole blood in response to TNF-α in patients and normal controls were also in the same range. Evaluation of both the absolute IFN-γ level and the stimulation indices of IFN-γ production by anti-CD3/anti-CD28-stimulated T-cell blasts in response to IL-12 and/or IL-18 revealed no abnormalities in patients compared with healthy controls (Table 4 and data not shown). Taken together, these data revealed no defects in type-1 immune responses in these five patients with previous pulmonary *M. kansasii* infection.

Table 3. IFN-γ production and enumeration of IFN-γ producing cells in response to mycobacterial antigens

	IFN-γ production (pg/mL)[a]					IFN-γ ELISPOT[b]
	PPD	MTB sonicate	STCF	*M. avium* sonicate	Ag85B	PPD
M. kansasii infection (n = 5)	622 ± 778	2892 ± 3995	683 ± 659	1572 ± 1806	490 ± 307	30 ± 28
M. marinum infection (n = 7)	761 ± 711	2421 ± 1173	1180 ± 805	2144 ± 1610	1060 ± 883	66 ± 71
Other non-tuberculous mycobacterial infection (n = 2)	490 ± 692	1205 ± 1045	462 ± 653	1242 ± 484	117 ± 88	47 ± 47
Tuberculosis (n = 4)	2256 ± 995[c]	3494 ± 1155[d]	3302 ± 1611[e]	2285 ± 1074	546 ± 231	140 ± 134
Healthy controls (n = 5)	28 ± 15	1017 ± 563	203 ± 172	912 ± 499	329 ± 172	22 ± 18

ELISPOT, enzyme-linked immunospot assay; MTB, *M. tuberculosis*; PPD, purified protein derivative; STCF, short-term culture filtrate of *M. tuberculosis*.
[a]After 6 days of culture.
[b]After 20 h of culture. ELISPOT results are adjusted for background values and expressed as mean spot-forming units $(SFU)_{antigen}$ − (mean SFU + (3 × SEM))$_{medium}$ [18]. Positive values indicate a positive response.
[c]p 0.02 for comparison with *M. kansasii* and with healthy controls.
[d]p 0.02 for comparison with healthy controls.
[e]$p < 0.03$ for comparison with each of the other groups.

Table 4. Evaluation of type-1 cellular immunity

	Response to LPS[a]			Response to IFN-γ[b]	
	TNF-α production (pg/mL)	IL-10 production (pg/mL)	CD64 up-regulation (ratio)	IFN-γ production (SI)	Response to IL-12[c]
Patients[d]					
Patient 5	1067	224	3.37		9.85
Patient 9	848	204	2.84		2.23
Patient 12	774	107	3.21		2.10
Patient 13	2429	347	2.84		1.82
Patient 16	576	605	2.47		1.85
Mean ± SD	1139 ± 742	297 ± 192	2.95 ± 0.35		3.57 ± 3.51
Controls				Controls	
C1	1277	230	3.19	C4	3.93
C2	2430	280	2.54	C5	3.94
C3	802	443	4.03	C6	2.00
Mean ± SD	1503 ± 837	318 ± 111	3.25 ± 0.75		3.29 ± 1.12
Other patients with pneumonia caused by non-tuberculous mycobacteria[e]					
	n = 5	*n* = 5	*n* = 7		*n* = 6
Mean ± SD	646 ± 395	230 ± 194	4.14 ± 1.72		4.42 ± 1.76
Controls	*n* = 5	*n* = 5	*n* = 7	Controls	*n* = 6
Mean ± SD	616 ± 287	157 ± 134	3.88 ± 1.73		5.22 ± 2.54

[a]TNF-α and IL-10 production of whole blood in response to 10 ng/mL lipopolysaccharide (LPS).
[b]CD64 expression on monocytes is expressed as the ratio of the median fluorescence of stimulated (100 U/mL of IFN-γ) over unstimulated cells.
[c]IL-12-mediated IFN-γ production by anti-CD3/anti-CD28-treated T-cell blasts is expressed as the ratio of IFN-γ production by cells in the presence or absence of IL-12 (1 ng/mL). SI, stimulation index. The healthy controls in this assay differ from the ones used in the assays analysing the LPS and IFN-γ responses.
[d]Numbering according to Table 1.
[e]These patients were from other regions in The Netherlands; their clinical data are not described in the present paper. The causative species were: *M. abscessus* (*n* = 1), *M. avium-intracellulare* (*n* = 3), *M. kansasii* (*n* = 2), and *M. malmoënse* (*n* = 1).

For comparison, the mean results for seven other patients, who lived in different regions within The Netherlands and had been diagnosed with *M. kansasii* pneumonia or isolated pulmonary infection caused by another non-tuberculous mycobacterial species, and for the corresponding controls are shown in Table 4. These patients had been referred to Leiden University Medical Center for evaluation of the type-1 immune system. As in the five patients from the present study, no abnormalities or deficiencies in type-1 immunity were identified.

DISCUSSION

The outcome of infection with *M. kansasii* is determined by the complex interplay between the characteristics of the host, the pathogen and the environment. While host susceptibility to non-tuberculous mycobacteria is a prominent feature of patients with defects in the cellular immune system [23–25], the factors that contribute most to the pathogenesis of *M. kansasii* pneumonia in apparently healthy persons are not known.

The clinical presentation of *M. kansasii* pneumonia in the patients described in this study varied from completely asymptomatic to extensive lung destruction accompanied by systemic symptoms. Most patients had bilateral infiltrates and/or cavitation, similar to the findings in previous studies [26], but the severity and duration of symptoms did not correlate with the extent of radiographic abnormalities. At least one of the risk factors recognised previously, such as chronic lung disease or a history of smoking, was present in all but two patients, indicating that abnormal lung anatomy or function is associated with susceptibility to invasive infection with *M. kansasii*, but may not be essential. The observed characteristics underscore the similarities between *M. kansasii*-induced pneumonia and classical tuberculosis.

With regard to the role of *M. kansasii* in pathogenesis, it was recognised >40 years previously that *M. kansasii* is a heterogeneous species [27]. More recently, five subtypes have been identified by genotyping of the 16S–23S intergenic spacer region [28]. With some overlap, mainly in genotype MKA-II, which has been associated with infection in immunosuppressed or AIDS patients [29,30], clinical and environmental isolates are distinct genotypically, which suggests phenotypic differences that determine the ability to cause disease in humans. Genotype MKA-I is the most common type found among clinical isolates [28,30] and has been isolated only rarely

from the environment (unpublished data). Indeed, all patient isolates in this study, as well as all Australian clinical isolates, were of genotype MKA-I. More strikingly, the MPTR fingerprint patterns were identical. MPTR is considered a suitable method for investigating *M. kansasii* isolates, as it gives distinct patterns in isolates of different origin, whereas other typing methods result in identical fingerprints [16]. However, MPTR does not differentiate between genotype MKA-I isolates from different sources, since all MKA-I isolates shared the same MPTR fingerprint pattern. In accordance with this finding, identical genotypes (by analysis of large-restriction-fragment polymorphisms) were found in 11 patients in an industrial zone in Japan [31]. Together, these data support the view that one genotype or genetic lineage of *M. kansasii* can be pathogenic for non-immunocompromised hosts. Interestingly, all isolates obtained from patients colonised with *M. kansasii* were also of genotype MKA-I or MKA-II, i.e., those associated with invasive infection. This observation strongly supports the hypothesis that colonisation and invasive infection represent two different phases of the same infection.

Large geographical differences in the incidence of *M. kansasii* pneumonia exist, but it is unclear whether this results from the spatially selective presence of the pathogen or circumstances leading to effective transmission. An association between dusty occupations and *M. kansasii* infections has been demonstrated [32]. The prevalence of (previously) heavily industrialised regions suggests a role for air quality. In the present study, only one patient was a former mine employee with silicosis and chronic lung disease.

In view of the fact that water systems are the presumed reservoir of *M. kansasii*, it is interesting that the profession of several patients involved intensive contact with water systems. It has not been demonstrated formally whether human infection with *M. kansasii* originates from artificial or natural sources. Presumably, *M. kansasii* bacteria isolated today underwent long-term evolutionary divergence and existed long before there were artificial water systems, and it is therefore reasonable to assume that a true natural reservoir exists. *M. kansasii* has been isolated, albeit rarely, from natural water reservoirs, soil and animal sources [33–36]. *M. kansasii* is a rare pathogen in animals, but manifest infection has been diagnosed in various animal species [36,37], including cattle (J. Pollock, personal communication). Most of the patients with *M. kansasii* pneumonia had regular and intensive contact with various animal species, but no common exposure could be identified. Thus far, there is no proof of zoonotic transmission. It is generally assumed that the route of infection is by inhalation, but without knowledge of the precise source of infection, nothing can be said about the circumstances leading to formation of *M. kansasii*-containing particles of an infective size. This lack of knowledge precludes preventive measures.

It is not known why patients who expectorate high numbers of *M. kansasii* in their sputum do not transmit the infection. An explanation could be that these are killed effectively by the innate immune system. Secondary cases of *M. kansasii* pneumonia have been described sporadically [38], but true human-to-human transmission would be impossible to prove, since a close contact who develops *M. kansasii* pneumonia as an apparent secondary case has, by definition, been exposed to the same environment harbouring the unrecognised source. Isolation of source patients and contact investigations are not indicated, but it would take several weeks from the time a patient presents with smear-positive pulmonary disease before *M. kansasii* could be identified. The application of molecular diagnostic methods, such as a PCR for the *M. tuberculosis* complex or for bacterial DNA of environmental mycobacteria [39,40], may lead to a more rapid and specific diagnosis and prevent the unnecessary costs and turmoil that come with the suspicion of contagious tuberculosis.

In the five available patients, including those without known risk factors, innate and antigen-specific cellular immune responses were studied in some detail, but no differences were found compared with control subjects. It cannot be concluded definitively from these findings that antigen-specific immune responses were normal, because these were tested after an interval ranging from 0 to 10 years (average 7 years) following diagnosis, and thus may not reflect the immune status at the time of active disease. However, tuberculin skin test results obtained at the time of diagnosis were positive in six of the ten patients who underwent skin testing, thereby indicating a capacity to display an adequate

delayed-type hypersensitivity response. In a previous study of patients with *M. kansasii* disease, 16 (59%) of 27 patients had a positive tuberculin skin test response [41].

The evaluation of the innate immune system, which can be assumed to reflect an intrinsic and unchanging characteristic of an individual, also revealed no differences compared with healthy control subjects. While more subtle or completely distinct immune defects could exist that escaped detection by the methods used, none of these patients had any unusual infection before or after diagnosis of *M. kansasii* pneumonia. Increasing numbers of cytokines, cytokine receptors and intracellular signal-transducing molecules are recognised as contributing to type-1 immunity, which is essential for resistance against intracellular pathogens such as mycobacteria (e.g., IL-5, IL-23, IL-27), and these could be candidates for further study.

The identical genotype and MPTR patterns of the isolates, and an absence of defects in host defence, suggest that a certain genotype of *M. kansasii* possesses virulence factors underlying its ability to cause disease in subjects without immune defects. Thus far, no virulence factors of *M. kansasii* have been described. Interestingly, *M. kansasii* and *M. marinum* share with *M. tuberculosis* the genes coding for ESAT-6 and CFP-10 [42–45]. These small proteins are immunodominant antigens encoded by the RD1 genomic region of *M. tuberculosis* and *M. bovis*, which was deleted at an early stage during the attenuation of *M. bovis* to the vaccine strain *M. bovis* Bacille Calmette Guérin (BCG) [42,46]. Recent studies showed that deletion of the *esat-6* gene in *M. bovis* [47] or deletion of RD1 in *M. tuberculosis* resulted in decreased virulence [48], while reinsertion of RD1 into BCG or *M. microti* increased virulence [49] in experimental animal models, which is in agreement with the notion that RD1-encoded proteins such as ESAT-6 and CFP-10 contribute to virulence. The function of these proteins is not known, but CFP-10 was recently demonstrated to have immunomodulating effects on mononuclear cells *in vitro* through effects on TNF-α production and nitric oxide synthesis [50]. It is not known whether the *esat-6* and *cpf-10* genes are present in all *M. kansasii* genotypes, clinical and environmental alike, or whether they are always expressed.

In conclusion, a study of the innate and adaptive immune systems of 17 patients from a limited geographical area who were diagnosed with *M. kansasii* pneumonia revealed none of the abnormalities in the type-1 immune response that have been associated previously with infection by non-tuberculous mycobacteria and salmonellae. The causative strains had identical genotypes and MPTR fingerprint patterns, but an epidemiologial connection between these patients or a common factor of exposure could not be identified. These findings are compatible with the presence of virulent strains of *M. kansasii* in geographically confined environmental sources in a setting amenable to effective transmission to humans.

ACKNOWLEDGEMENTS

All patients and control subjects are gratefully acknowledged for their cooperation. We thank C. Prins, who assisted with obtaining informed consent and blood sampling. A. van Wengen and T. de Boer are thanked for expert assistance with the immunological evaluation. B. Dwyer kindly provided the *M. kansasii* isolates from Australian patients.

Financial support for this study was obtained from the European Commission (EC Grant 2000-0630 on Molecular Epidemiology of Tuberculosis), Netherlands Leprosy Foundation and Netherlands Organisation for Scientific Research (Nederlandse Organisatie voor Wetenschappelijk Onderzoek).

REFERENCES

1. Wolinsky E. Mycobacterial diseases other than tuberculosis. *Clin Infect Dis* 1992; **15**: 1–10.
2. Evans AJ, Crisp AJ, Hubbard RB, Colville A, Evans SA, Johnston ID. Pulmonary *Mycobacterium kansasii* infection: comparison of radiological appearances with pulmonary tuberculosis. *Thorax* 1996; **51**: 1243–1247.
3. Evans SA, Colville A, Evans AJ, Crisp AJ, Johnston ID. Pulmonary *Mycobacterium kansasii* infection: comparison of the clinical features, treatment and outcome with pulmonary tuberculosis. *Thorax* 1996; **51**: 1248–1252.
4. Falkinham JO. Epidemiology of infection by nontuberculous mycobacteria. *Clin Microbiol Rev* 1996; **9**: 177–215.
5. Hoffner SE. Pulmonary infections caused by less frequently encountered slow-growing environmental mycobacteria. *Eur J Clin Microbiol Infect Dis* 1994; **13**: 937–941.
6. Bittner MJ, Horowitz EA, Safranek TJ, Preheim LC. Emergence of *Mycobacterium kansasii* as the leading mycobacterial pathogen isolated over a 20-year period at a midwestern Veterans Affairs hospital. *Clin Infect Dis* 1996; **22**: 1109–1110.
7. Kubin M, Svandova E, Medek B, Chobot S, Olsovsky Z. *Mycobacterium kansasii* infection in an endemic area of Czechoslovakia. *Tubercle* 1980; **61**: 207–212.

8. Appelman AC, Roelfsema J. Pneumoconiosis in workers of the Dutch coal mines; a retrospective view in relation to the closing of the mines [Pneumoconiose bij arbeiders in Nederlandse steenkolenmijnen; een terugblik met betrekking tot de mijnsluitingen]. *Ned Tijdschr Geneeskd* 1979; **123**: 2131–2135.
9. Lillo M, Orengo S, Cernoch P, Harris RL. Pulmonary and disseminated infection due to *Mycobacterium kansasii*: a decade of experience. *Rev Infect Dis* 1990; **12**: 760–767.
10. Engel HWB, Berwald LG, Havelaar AH. The occurrence of *Mycobacterium kansasii* in tapwater. *Tubercle* 1980; **61**: 21–26.
11. Ottenhoff TH, Verreck FA, Lichtenauer-Kaligis EG, Hoeve MA, Sanal O, van Dissel JT. Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. *Nat Genet* 2002; **32**: 97–105.
12. Casanova JL, Abel L. Genetic dissection of immunity to mycobacteria: the human model. *Annu Rev Immunol* 2002; **20**: 581–620.
13. Breathnach A, Levell N, Munro C, Natarajan S, Pedler S. Cutaneous *Mycobacterium kansasii* infection: case report and review. *Clin Infect Dis* 1995; **20**: 812–817.
14. Cerdá de Palou E, Sulzer MA, van Noord JA. Indications for an increase in lung disease caused by *Mycobacterium kansasii* in the former Limburg mining area? [Aanwijzingen voor toename van longziekte door *Mycobacterium kansasii* in de voormalige Limburgse mijnstreek?] *Ned Tijdschr Geneeskd* 1995; **139**: 1231–1236.
15. Anonymous. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. *Am J Respir Crit Care Med* 1997; **156**(suppl): S1–S25.
16. Hermans PW, van Soolingen D, van Embden JD. Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae*. *J Bacteriol* 1992; **174**: 4157–4165.
17. Suffys PN, da Silva RA, de Oliveira M *et al.* Rapid identification of Mycobacteria to the species level using INNO-LiPA Mycobacteria, a reverse hybridization assay. *J Clin Microbiol* 2001; **39**: 4477–4482.
18. Arend SM, Van Meijgaarden KE, de Boer K *et al.* Tuberculin skin testing and in vitro T cell responses to ESAT-6 and culture filtrate protein 10 after infection with *Mycobacterium marinum* or *M. kansasii*. *J Infect Dis* 2002; **186**: 1797–1807.
19. de Jong R, Altare F, Haagen I-A *et al.* Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. *Science* 1998; **280**: 1435–1438.
20. Verhagen CE, de Boer T, Smits HH *et al.* Residual type 1 immunity in patients genetically deficient for interleukin 12 receptor beta1 (IL-12Rbeta1): evidence for an IL-12Rbeta1-independent pathway of IL-12 responsiveness in human T cells. *J Exp Med* 2000; **192**: 517–528.
21. Lichtenauer-Kaligis EG, de Boer T, Verreck FA *et al.* Severe *Mycobacterium bovis* BCG infections in a large series of novel IL-12 receptor beta1 deficient patients and evidence for the existence of partial IL-12 receptor beta1 deficiency. *Eur J Immunol* 2003; **33**: 59–69.
22. Harth G, Lee B-Y, Wang J, Clemens DL, Horwitz MA. Novel insights into the genetics, biochemistry, and immunocytochemistry of the 30-kilodalton major extracellular protein of *Mycobacterium tuberculosis*. *Infect Immun* 1996; **64**: 3038–3047.
23. Witzig RS, Fazal BA, Mera RM *et al.* Clinical manifestations and implications of coinfection with *Mycobacterium kansasii and* human immunodeficiency virus type 1. *Clin Infect Dis* 1995; **21**: 77–85.
24. Anzalone G, Cei M, Vizzaccaro A, Tramma B, Bisetti A. *M. kansasii* pulmonary disease in idiopathic CD4+ T-lymphocytopenia. *Eur Respir J* 1996; **9**: 1754–1756.
25. Jacobson KL, Teira R, Libshitz HI *et al. Mycobacterium kansasii* infections in patients with cancer. *Clin Infect Dis* 2000; **30**: 965–969.
26. Rauscher CR, Kerby G, Ruth WE. A ten-year clinical experience with *Mycobacterium kansasii*. *Chest* 1974; **66**: 17–19.
27. Wayne LG. Two varieties of *Mycobacterium kansasii* with different clinical significance. *Am Rev Resp Dis* 1962; **86**: 651–656.
28. Alcaide F, Richter I, Bernasconi C *et al.* Heterogeneity and clonality among isolates of *Mycobacterium kansasii*: implications for epidemiological and pathogenicity studies. *J Clin Microbiol* 1999; **35**: 1959–1964.
29. Tortoli E, Simonetti MT, Lacchini C, Penati V, Urbano P. Tentative evidence of AIDS-associated biotype of *Mycobacterium kansasii*. *J Clin Microbiol* 1994; **32**: 1779–1782.
30. Taillard C, Greub G, Weber R *et al.* Clinical implications of *Mycobacterium kansasii* species heterogeneity: Swiss National Survey. *J Clin Microbiol* 2003; **41**: 1240–1244.
31. Iinuma Y, Ichiyama S, Hasegawa Y, Shimokata K, Kawahara S, Matsushima T. Large-restriction-fragment analysis of *Mycobacterium kansasii* genomic DNA and its application in molecular typing. *J Clin Microbiol* 1997; **35**: 596–599.
32. British Thoracic and Tuberculosis Association. Opportunist mycobacterial pulmonary infection and occupational dust exposure: an investigation in England and Wales. *Tubercle* 1975; **56**: 295–313.
33. Powell BL, Steadham JE. Improved technique for isolation of *Mycobacterium kansasii* from water. *J Clin Microbiol* 1981; **13**: 969–975.
34. Collins CH, Grange JM, Yates MD. Mycobacteria in water. *J Appl Bacteriol* 1984; **57**: 193–211.
35. Chapman JS, Bernard JS, Speight M. Isolation of mycobacteria from raw milk. *Am Rev Resp Dis* 1965; **91**: 351–355.
36. Bercovier H, Vincent V. Mycobacterial infections in domestic and wild animals due to *Mycobacterium marinum*, *M. fortuitum*, *M. chelonae*, *M. porcinum*, *M. farcinogenes*, *M. smegmatis*, *M. scrofulaceum*, *M. xenopi*, *M. kansasii*, *M. simiae and M. genavense*. *Rev Sci Tech* 2001; **20**: 265–290.
37. Acosta B, Real F, Ferrer O, Deniz S, Poveda B. Isolation of *Mycobacterium kansasii* from a tuberculin-positive goat. *Vet Rec* 1998; **142**: 195–196.
38. Penny ME, Cole RB, Gray J. Two cases of *Mycobacterium kansasii* infection occurring in the same household. *Tubercle* 1982; **63**: 129–131.
39. Richter E, Niemann S, Rusch-Gerdes S, Hoffner S. Identification of *Mycobacterium kansasii* by using a DNA probe (AccuProbe) and molecular techniques. *J Clin Microbiol* 1999; **37**: 964–970.
40. Ross BC, Jackson K, Yang M, Sievers A, Dwyer B. Identification of a genetically distinct subspecies of *Mycobacterium kansasii*. *J Clin Microbiol* 1992; **30**: 2930–2933.
41. Rauscher CR, Kerby G, Ruth WE. *Mycobacterium kansasii* in Kansas: saprophyte or infection? *Chest* 1974; **66**: 162–164.
42. Harboe M, Oettinger T, Wiker HG, Rosenkrands I, Andersen P. Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacte-*

rium bovis and for its absence in *Mycobacterium bovis* BCG. *Infect Immun* 1996; **64**: 16–22.
43. Pollock JM, Andersen P. The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. *J Infect Dis* 1997; **175**: 1251–1254.
44. Skjøt RLV, Oettinger T, Rosenkrands I *et al*. Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. *Infect Immun* 2000; **68**: 214–220.
45. Colangeli R, Spencer JS, Bifani P *et al*. MTSA-10, the product of the Rv3874 gene of *Mycobacterium tuberculosis*, elicits tuberculosis-specific, delayed-type hypersensitivity in guinea pigs. *Infect Immun* 2000; **68**: 990–993.
46. Behr MA, Wilson MA, Gill WP *et al*. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 1999; **284**: 1520–1523.
47. Wards BJ, de Lisle GW, Collins DM. An *esat6* knockout mutant of *Mycobacterium bovis* produced by homologous recombination will contribute to the development of a live tuberculosis vaccine. *Tuber Lung Dis* 2000; **80**: 185–189.
48. Lewis KN, Liao R, Guinn KM *et al*. Deletion of RD1 from *Mycobacterium tuberculosis* mimics Bacille Calmette-Guérin attenuation. *J Infect Dis* 2003; **187**: 117–123.
49. Pym AS, Brodin P, Brosch R, Huerre M, Cole ST. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol Microbiol* 2002; **46**: 709–717.
50. Trajkovic V, Singh G, Singh B, Singh S, Sharma P. Effect of *Mycobacterium tuberculosis*-specific 10-kilodalton antigen on macrophage release of tumor necrosis factor alpha and nitric oxide. *Infect Immun* 2002; **70**: 6558–6566.